ENGINEERED OPEN READING FRAME FOR P53

[01] This invention was made with government support under grant number CA 81511 awarded by the National Institutes of Health/National Cancer Institute. The government has certain rights to the invention.

FIELD OF THE INVENTION

[02] The invention is related to the field of cancer therapy. More particularly, it is related to the area of studying the *p53* gene and development of therapeutics related to cancers containing *p53* mutations.

BACKGROUND OF THE INVENTION

[03] **p53 inactivation and cancer.** The tumor suppressor gene *p53* is of central importance for the genetic stability of human cells (Donehower and Bradley, 1993; Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). The p53 protein is active as a homo-tetramer and exerts its tumor suppressor function mainly as a transcription factor that induces G1 and G2 cell cycle arrest and/or apoptosis (Donehower and Bradley, 1993; Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997; Hermeking et al., 1998). The p53-mediated G1 arrest is its best characterized activity and involves transcriptional activation of the downstream gene $p21^{WAF1/CIP1/SDI1}$ (Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). Other downstream effector genes for p53-mediated G1 arrest may exist, since $p21^{-/-}$ mouse embryonic fibroblasts do not show complete abrogation of G1 arrest after DNA damage (Brugarolas et al., 1995; Deng et al., 1995). The G2/M block mediated by p53 involves, at least in part, induction of *14-3-3*σ (Hermeking et al., 1998).

[04] The mechanisms for apoptosis induction and their relative importance remain less clear at present. In certain settings p53 clearly induces pro-apoptotic genes. These include *BAX* and *Fas/APO1* (Miyashita and Reed, 1995; Owen-Schaub et al., 1995) neither of which, however, is an absolute requirement for p53-induced apoptosis (Knudson et al., 1995; Fuchs

et al., 1997; Yin et al., 1997). Recently, many more genes have been identified that are induced directly or indirectly during p53-mediated apoptosis (Polyak et al., 1997; Wu et al., 1997; Yin et al., 1998), but the essential genes for p53-induced apoptosis still have to be determined. Transcriptional repression of anti-apoptotic genes, such as *bcl-2*, may play a role (Haldar et al., 1994; Miyashita et al., 1994) and other non-transcriptional mechanisms may be important as well (Caelles et al., 1994; Wagner et al., 1994; Haupt et al., 1995; Wang et al., 1996; White, 1996).

[05] Several upstream signals activate p53. These include DNA damage, hypoxia and critically low ribonucleoside triphosphate pools (Kastan et al., 1991; Graeber et al., 1996; Linke et al., 1996). Once activated, p53 induces either cell cycle arrest or apoptosis, depending on several factors such as the amount of DNA damage, cell type and cellular milieu, *e.g.,* presence or absence of growth factors (Donehower and Bradley, 1993; Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997).

[06] Cancer cells show decreased fidelity in replicating their DNA, often resulting in DNA damage, and tumor masses have inadequate neovascularization leading to ribonucleoside triphosphate or oxygen deprivation, all upstream signals that activate p53. In view of p53's capability to induce cell cycle arrest or apoptosis under these conditions it is not surprising that absent or significantly reduced activity of the tumor suppressor protein p53 is a characteristic of more than half of all human cancers (Hollstein et al., 1991; Harris and Hollstein, 1993; Greenblatt et al., 1994). In the majority of cancers, p53 inactivation is caused by missense mutations in one *p53* allele, often with concomitant loss-of-heterozygosity (Michalovitz et al., 1991; Vogelstein and Kinzler, 1992; Donehower and Bradley, 1993; Levine, 1997). These mutations affect almost exclusively the core DNA-binding domain of p53 that is responsible for making contacts with p53 DNA-binding sites (Cho et al., 1994), while mutations in the N-terminal transactivation domain or the C-terminal tetramerization domain are extremely rare (Figure 1) (Beroud and Soussi, 1998; Cariello et al., 1998; Hainaut et al., 1998). Contrary to wild-type p53, p53 cancer mutants have a long half-life and accumulate to high levels in cancer cells (Donehower and Bradley, 1993; Lowe, 1995). This may be explained by their inability to activate the *MDM-2* gene (Lane and Hall, 1997), since mdm-2 induces degradation of p53 via the ubiquitin pathway as part of a negative feedback loop (Haupt et al., 1997; Kubbutat et al., 1997). The unusually high frequency of *p53*

missense mutations in human cancers (as opposed to mutations resulting in truncated proteins) is explained by their dominant-negative effect that depends on the intact C-terminal tetramerization domain. The C-terminus allows p53 cancer mutants to form hetero-tetramers with wild-type p53 (Milner and Medcalf, 1991), thus reducing, or even abrogating, the activity of wild-type p53 protein (Michalovitz et al., 1991; Vogelstein and Kinzler, 1992; Hann, 1995; Brachmann et al., 1996; Ko and Prives, 1996). In addition, there is evidence that at least some of the same missense mutations may confer a gain-of-function (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997).

[07] p53 abnormalities and cancer therapy. Considering the activities of the p53 tumor suppressor protein, reconstitution of wild-type p53 activity to cancers would be of large therapeutic benefit, an idea that is supported by several lines of evidence from epidemiological, clinical and basic cancer research (Fisher, 1994; Lowe, 1995; Harris, 1996a).

[08] Several human malignancies that are usually diagnosed at a young age, such as testis cancer, pediatric acute lymphoblastic leukemia and Wilms tumor, can be successfully eradicated even at advanced stages. They all have in common that they carry wild-type *p53* (Heimdal et al., 1993; Wada et al., 1993; Malkin et al., 1994). At the same time, subgroups of these malignancies with a poor prognosis, for example the anaplastic variant of Wilms tumor, commonly do carry *p53* mutations (Bardeesy et al., 1995). Similarly, tumor types that are often resistant to conventional therapies and difficult to treat at advanced stages, such as lung, prostate, colorectal, breast, head and neck, pancreatic and gastric cancers, show a high frequency of *p53* mutations (Hollstein et al., 1991; Fisher, 1994; Lowe, 1995; Harris, 1996a; Beroud and Soussi, 1998; Cariello et al., 1998; Hainaut et al., 1998).

[09] These findings have spurred great interest in exploring p53 as a predictive marker for response to therapy and for overall prognosis. The majority of cancer types have been evaluated to some extent, and the publications are too numerous to be summarized here. As an example, studies in breast, head and neck, lung and ovarian cancers have found a good correlation between p53 abnormalities and poor survival and poor response to therapy (Thor et al., 1992; Allred et al., 1993; Bergh et al., 1995; Rusch et al., 1995; Sauter et al., 1995; Righetti et al., 1996; Berns et al., 1998; Huang et al., 1998). The results are not always unequivocal, as some studies were unable to detect a statistically significant difference between cancers with and without functional p53 (Isola et al., 1992; Elledge et al., 1995).

These discrepancies may be due to confounding factors. For example, a cancer with a poor prognosis because of degradation of p53 by overexpressed mdm-2 may be incorrectly scored as a cancer with functional p53 if the mdm-2 status of the cancer is not evaluated. In addition, the sample size of many studies was not large enough to make firm conclusions.

[10] Strong evidence for a central role of p53-mediated apoptosis in cancer therapy is provided by experiments in cell lines with and without functional p53. Comparison of wild-type and p53-deficient thymocytes established that p53 is required for radiation- and etoposide-induced apoptosis (Clarke et al., 1993; Lowe et al., 1993a). Similar experiments in adenovirus *E1A* transformed mouse embryo fibroblasts showed that apoptosis induced by radiation, 5-fluorouracil, etoposide and adriamycin also depends on functional p53 in these cells (Lowe et al., 1993b). These studies were extended into a mouse model where again only tumors with functional p53 showed good treatment responses to radiation and adriamycin, while p53-negative tumors were highly resistant to therapy and showed little evidence of apoptosis (Lowe et al., 1994). Results of the Developmental Therapeutics Program of the NCI impressively and independently confirmed these findings. An analysis of the cytostatic and cytotoxic effects of 123 compounds on 60 different human cancer cell lines showed a very good correlation between *p53* mutations and resistance to many commonly used chemotherapeutic agents (O'Connor et al., 1997; Weinstein et al., 1997). All these data do not necessarily indicate that functional p53 is absolutely essential for chemotherapy-induced apoptosis. In fact, chemotherapy drugs can kill cancer cells through p53-independent mechanisms (Kaufmann, 1989; Strasser et al., 1994; Bracey et al., 1995). The sum of the evidence, however, suggests that cancer agents are significantly more effective in the presence of p53 (Fisher, 1994; Lowe, 1995; Harris, 1996a).

[11] Based on the discussed studies and the general knowledge about p53, p53 and its pathways have been recognized as a prime target for developing new cancer therapies (Fisher, 1994; Gibbs and Oliff, 1994; Kinzler and Vogelstein, 1994; Lowe, 1995; Milner, 1995; Harris, 1996a). In particular, the high frequency of *p53* mutations in cancers makes therapeutic strategies for restoring this tumor suppressor pathway highly desirable since a large number of patients could potentially benefit. It has been estimated that every year approximately 330,000 patients in the United States and 2.4 million patients worldwide are diagnosed with cancers that contain *p53* mutations (Harris, 1996a, 1996b).

[12] Strategies to partially or completely restore wild-type p53 function to cancer cells. Restoration of wild-type p53 activity to cancer cells is the most direct way of making cancer cells more susceptible to apoptosis and can be pursued in two ways. The first strategy is to reintroduce wild-type p53, perhaps by gene therapy (Roth et al., 1996; Barinaga, 1997; Nielsen and Maneval, 1998), and does not rely on the p53 status of a given cancer. The current major challenge is efficient and selective targeting of wild-type *p53* expression constructs to the cancerous cells (Nielsen and Maneval, 1998). A major drawback of this approach is that it may be less effective for cancers with vast amounts of a dominant-negative p53 cancer mutant. This strategy would be greatly aided by the availability of p53 proteins that are resistant to the dominant-negative effects of p53 cancer mutants and that are superior to wild-type p53 in inducing apoptosis, classes of p53 proteins that to date have not been described.

[13] The second strategy is only possible because of the unique pattern of *p53* missense mutations in human cancers and aims at therapeutically exploiting the abundant p53 mutant protein found in many cancers. Since the resulting p53 cancer mutants are full-length proteins each with a structurally altered core domain, but an intact transactivation domain and an intact C-terminal tetramerization domain, one could restore wild-type activity to the p53 cancer mutants in these tumors (Gibbs and Oliff, 1994; Lowe, 1995; Milner, 1995; Harris, 1996a). This can be achieved in at least two ways. One attempt has been to interfere with the extreme C-terminal autoregulatory domain of p53 by using antibodies (Halazonetis and Kandil, 1993; Hupp et al., 1993; Abarzua et al., 1995; Niewolik et al., 1995) or peptides spanning part of this region (Hupp et al., 1995; Abarzua et al., 1996; Selivanova et al., 1997). This strategy presumably activates p53 cancer mutants by blocking the ability of the very C-terminus to fold back onto and inhibit the p53 core domain. It could succeed with p53 cancer mutants that retain residual activity and which only require additional activation to exceed the threshold required for biological effects. However, antibodies and peptides clearly cannot be delivered efficiently to cancer cells in patients (Selivanova et al., 1997). Small molecule compounds with similar effects could overcome this problem, but their design is currently not feasible since the exact structural basis of this negative autoregulation and of its neutralization by antibodies or peptides is not known due to lack of a crystal structure for the full-length p53 protein (Ko and Prives, 1996; Selivanova et al., 1997). In addition, this approach may activate mutant and wild-type p53 proteins indiscriminately, thus possibly

causing significant side effects due to inappropriate wild-type p53-induced apoptosis in normal tissues.

[14] A more direct approach is to revert the effects of tumorigenic mutations on the structure and function of the p53 core domain itself by means of small molecules. This strategy is preferable since it is predicted to selectively stabilize p53 cancer mutants. It also holds the promise of restoring function to completely inactive p53 cancer mutants. Restoring the normal configuration to a p53 cancer mutant is considered more challenging than inhibiting the function of a protein by small molecules (Gibbs and Oliff, 1994). However, there are examples: small molecule compounds that bind the central cavity of the hemoglobin tetramer can act as allosteric effectors and stabilize the T state of hemoglobin over the R state (Abraham et al., 1992); and small molecule compounds that stabilize the transthyretin tetramer against dissociation can prevent amyloid fibril formation *in vitro* (Miroy et al., 1996). Furthermore, the technology of structure-based drug design is steadily advancing so that this challenge may be met (Bohacek et al., 1996; Marrone et al., 1997).

[15] ***p53* mutations and the p53 core DNA-binding domain.** These considerations make it clear that a detailed understanding of the structural consequences of *p53* cancer mutations on the p53 core domain is needed. More significantly, stabilizing mechanisms must be identified that can override the deleterious structural effects of *p53* cancer mutations.

[16] The crystal structure of the wild-type p53 core domain has given enormous insight into how p53 interacts with its DNA-binding sites (Cho et al., 1994). The structures of the C-terminal tetramerization domain and of the N-terminal transactivation domain (complexed to mdm-2) have been determined as well (Clore et al., 1994; Jeffrey et al., 1995; Kussie et al., 1996). The structure of the full-length protein as a homo-tetramer, however, is solely based on computer modeling (Jeffrey et al., 1995) and suggests that the core domain functions as a separate entity that is connected to the other domains through flexible linkers. The core domain spans 191 amino acids and consists of a β sandwich that serves as the scaffold for two large loops (termed L2 and L3) and a loop-sheet-helix motif. The loops and the loop-sheet-helix motif form the DNA-binding surface of p53 and provide contacts to the DNA backbone and the edges of the bases (Figure 1A). This structural organization was considered unique until the recent discovery of p73 made it clear that p53 is actually part of a family of transcription factors (Jost et al., 1997; Kaghad et al., 1997). The vast majority of tumor-derived *p53* missense mutations map to this core domain (Figure 1B) and invariably result in

the reduction or loss of DNA-binding. These cancer mutations are predicted to fall into two classes; one class of mutations maps to DNA-contacting residues and eliminates p53-DNA contacts (functional mutations); the other, larger class of mutations probably affects the structural integrity of the DNA-binding domain (structural mutations). These structural defects may range from small structural shifts to the global destabilization and unfolding of the p53 core domain. The most frequent *p53* cancer mutations affect amino acids that are part of important structures of the p53 core domain, such as the L3 loop and the loop-sheet-helix motif that provide DNA contacts. However, the high frequency of a mutation does not predict how deleterious its effects on the structural integrity of the core domain are, since the frequency of these mutations is also determined by exogenous carcinogens and endogenous biological processes (Donehower and Bradley, 1993; Greenblatt et al., 1994).

[17] To date, our understanding of the structural consequences of *p53* cancer mutations is limited to predictions using the structure of the wild-type p53 core domain, biochemical data (Cho et al., 1994) and experiments with monoclonal antibodies that recognize areas of the p53 core domain that are not accessible in the correctly folded state (Donehower and Bradley, 1993; Gottlieb and Oren, 1996; Levine, 1997). Similarly, very little is known about how the effects of cancer mutations can be overcome.

[18] There is a need in the art for the identification of small molecules and proteins that will restore function to mutant p53 proteins. Such small molecules and proteins will increase the ability of mutant p53 to induce cell cycle arrest and/or apoptosis. There is also a need in the art for reagents to aid in the development and identification of such p53 suppressors.

BRIEF SUMMARY OF THE INVENTION

[19] These and other objects of the invention are achieved by providing one or more of the embodiments described below. In one embodiment of the invention a non-naturally occurring nucleic acid molecule encoding wild-type human p53 protein is provided. The p53 protein has a sequence shown in SEQ ID NOs: 54-57. The nucleic acid employs a plurality of alternative codons to those present in naturally occurring wild-type human p53 coding sequence as shown in SEQ ID NO: 58-61. At least a portion of the alternative codons provides additional unique restriction sites to the human p53 coding sequence.

[20] In another embodiment of the invention a non-naturally occurring nucleic acid molecule is provided. The nucleic acid molecule employs a plurality of alternative codons to those present in naturally occurring wild-type human p53 coding sequence. The alternative codons do not cause amino acid changes from wild-type human p53. At least a portion of the alternative codons provides additional unique restriction sites to the human p53 coding sequence. The nucleic acid further comprises a *p53* mutation of a human cancer.

[21] In a further embodiment of the invention a non-naturally occurring nucleic acid molecule is provided. The nucleic acid molecule employs a plurality of alternative codons to those present in naturally occurring wild-type human p53 coding sequence. The alternative codons cause no amino acid changes from wild-type p53. At least a portion of the alternative codons provides additional unique restriction sites to the human p53 coding sequence. The nucleic acid further contains a mutation in a codon for a residue that is post-translationally modified in wild-type p53. The mutation prevents post-translational modification of the residue.

[22] These and other embodiments of the invention, which will be apparent to those of skill in the art, provide the art with reagents to develop p53 suppressors for the treatment of cancer. Vectors that contain these reagents exhibit better expression in host cells and are more amenable to manipulation to arrive at p53 suppressors for treatment of cancer. Gene therapy for treatment of cancers with *p53* mutations using these nucleic acids is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[23] Figure 1A and 1B. Structure of the p53 core DNA-binding domain and pattern of missense mutations within the core domain. Figure 1A shows the structure of the p53 core domain. A β sandwich serves as the scaffold for two large loops (termed L2 and L3) and a loop-sheet-helix motif. The loops and the loop-sheet-helix motif form the DNA binding surface of p53. The L3 loop makes DNA contacts in the minor groove, while the H2 α helix and the L1 loop of the loop-sheet-helix motif make contacts in the major groove. The L2 and L3 loops provide stability to the DNA-binding surface through interactions with a Zn atom (Cho et al., 1994.) Figure 1B is a map of tumor-derived p53 core domain mutations against a schematic p53 protein. The vast majority of tumor-derived *p53* missense mutations map to the p53 core domain as shown by the mutation histogram superimposed on the schematic p53

protein. Six "hot spot" codons are preferentially mutated due to exogenous carcinogens and endogenous biological processes. Mutations in the N-terminal transactivation and the C-terminal tetramerization domain are exceedingly rare. The white box in the very C-terminus indicates the location of the autoregulatory domain.

[24] Figure 2A, 2B, 2C, and 2D show the design and characterization of a new *p53* open reading frame. Figure 2A is a comparison of the *p53* open reading frame before and after cloning to introduce multiple restriction sites by silent mutagenesis. Before cloning of the new open reading frame, suppressor mutations with the most frequent *p53* cancer mutations required a significant amount of subcloning. Figure 2B shows that the new pTW500 expression plasmid (designer-*p53* gene) and pRB16 (native *p53* gene) have the same phenotype, $Ura^{+}Foa^{S}$, in a yeast strain with the reporter gene 1cUAS53::URA3. Figure 2C shows that pTW500 and pRB16 have equal growth in SC-Ura media. A control strain with the reporter gene alone does not grow, while a strain with the URA3 gene shows superior growth. Figure 2D shows that the new p53 expression plasmid pTW500 leads to similar p53 protein levels in yeast, as compared to the previously used pRB16.

[25] Figure 3 shows the p53 cancer mutation to be analyzed. The p53 cancer mutations were chosen by their relative frequency in human cancer. All eight cancer mutations that will be used for the search of suppressor mutations are located at "hot spot" codons, codons that have a particularly high frequency of mutations. The first set and the second set of mutations (chosen for the subcloning analysis) comprise approximately 37% of all human cancers with *p53* mutations. This is estimated to correspond to 123,000 cancer patients per year in the United States and 890,000 cancer patients worldwide (Harris, 1996a, 1996b.)

DETAILED DESCRIPTION OF THE INVENTION

[26] The inventor has discovered alternative *p53* open reading frames with amino acid sequences identical to a wild-type p53 amino acid sequence. The open reading frames optimize cloning with and expression of *p53* nucleotide sequences. The *p53* nucleotide sequences can be delivered by gene therapy vectors to human cancers containing *p53* mutations to optimize expression of wild-type p53. *P53* suppressor mutations can be readily cloned into the alternative *p53* open reading frames. Gene therapy vectors containing these sequences can be delivered to human cells containing a mutation in *p53*.

[27] A naturally occurring wild-type human p53 coding sequence may be any wild-type human p53 that is naturally found in humans and is characterized by wild-type p53 activity. Examples of such polymorphic human *p53* sequences can be found at the www host server, iarc.fr domain name, p53/Polymorphism.html#Table directory. Preferably the human p53 coding sequence has the sequence of GenBank Accession number NP_000537 (SEQ ID NO: 58). Common polymorphisms include GAT at codon 21, CCA at codon 36, CGG at codon 213, TCG at codon 47, and CCC at codon 72. One or more polymorphisms can be found in a single coding sequence. Most preferably the wild-type human p53 coding sequence is any of sequences SEQ ID NO: 58-61.

[28] Similarly, the wild-type human p53 protein sequence is the sequence of any p53 protein that exhibits wild-type human p53 activity. Preferably the p53 sequence is the sequence found at GenBank Accession number NP_000537 (SEQ ID NO: 55). If the wild-type human p53 sequence is the sequence of a different polymorphic form of p53, preferably it has a Ser at residue 47 (SEQ ID NO: 56) or Pro at residue 72 (SEQ ID NO: 54), or both (SEQ ID NO: 57).

[29] Non-naturally occurring alternative codons may be preferable for use in mammalian cells, yeast cells, bacterial cells, or any combination thereof. The alternative codons may also be more preferred for use in *Drosophila* cells. Any number of alternative codons may be introduced into the sequence of the wild-type human p53 coding sequence. Preferably at least 2, at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 75, at least 100, at least 120, at least 140, or at least 150 alternative codons may be introduced. The codons do not change the amino acid sequence of the p53 protein. The alternative codons can be introduced for any known purpose in the art. The alternative codons may be introduced to insert a new restriction enzyme cleavage site into the open reading frame, to delete a restriction enzyme cleavage site from the open reading frame, to produce a polymorphic p53 found in the human population that does not change the p53 amino acid sequence, or to optimize expression of the *p53* nucleic acids in a particular organism.

[30] At least a portion of the alternative codons provides additional unique restriction sites to the human *p53* sequence. A portion is any percentage of the total number of alternative codons introduced into the *p53* sequence. The portion may be at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 75%, 80%, 90%, 95%, or 99% of all the alternative codons

introduced into the p53 encoding nucleic acids. The non-naturally occurring *p53* nucleic acids may contain at least 1, at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 75, or at least 100 additional restriction sites as a result of introducing alternative codons.

[31] Preferably the nucleic acid will have the nucleotide sequence of one of the three artificial *p53* open reading frames as shown in SEQ ID NO: 1-3. The nucleic acid sequence may also contain a polymorphism common in the human population. SEQ ID NO: 62-64 are polymorphic variants of SEQ ID NO: 1. SEQ ID NO: 65-67 are polymorphic variants of SEQ ID NO: 2. SEQ ID NO: 68-70 are polymorphic variants of SEQ ID NO: 3.

[32] The nucleic acids of the invention can be deoxyribonucleic acids (DNA) or ribonucleic acid (RNA) molecules such as mRNA. The nucleic acids can be linear nucleic acids or they can be cloned into any suitable vector. Suitable vectors include plasmids, artificial chromosomes, or viral genomes. Plasmids are well known in the art and include plasmids that are suitable for introduction of the *p53* gene into bacterial, yeast, mammalian, insect, or other eukaryotic cells or organisms. The plasmids may be available through a commercial vendor, or may be noncommercial plasmids, or derivatives thereof. Artificial chromosomes are preferably the artificial chromosomes of humans, yeast, or bacteria. Viral vectors are also well known in the art. Preferably the viral genome is the genome of an adeno-associated virus, adenovirus, herpes virus, retrovirus, or vaccinia virus. Viral vectors such as Baculovirus may also be used for subsequent use in insect cells.

[33] p53-encoding nucleic acids can be introduced into a vector by any technique known for this purpose. Several nonlimiting examples of such techniques include restriction enzyme digestion of the *p53* nucleic acids and direct ligation into a vector, or PCR amplification of the *p53* nucleic acids and subsequent cloning by restriction enzyme digestion and ligation into a vector. Other techniques for cloning the *p53* nucleic acids into a vector can be found in Sambrook, J., Fritsch, E. and Maniatis, T., *Molecular Cloning: A Laboratory Manual* Second Edition. Cold Spring Harbor Press, Cold Spring Harbor, NY, and Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*. Wiley, Interscience New York (1987). Methods of mutagenesis of the *p53* nucleic acids can also be found in these references.

[34] It is also contemplated that the nucleic acid encoding an alternative *p53* open reading frame can further comprise regulatory sequences that enhance the expression of the *p53* gene.

Promoters and enhancers are well known to those of skill in the art. Several nonlimiting examples include constitutive promoters such as the strong promoters of cytomegalovirus, SV40, or Rous sarcoma virus. Promoters can also be inducible promoters that are induced by drugs like tetracycline, or tissue specific promoters. Tissue specific promoters include the albumin promoter for expression in the liver, the myosin light chain 1 promoter for expression in muscle and endothelial cells, the surfactant protein A or keratin 18 for expression in lung, and the prostate specific antigen (PSA) promoter, the probasin (PB) promoter, or the prostate specific membrane antigen promoter for expression in prostate. Tumor specific promoters can also be used. Tumor specific promoters include the tyrosine kinase promoter for B16 melanomas, the DF3/MUC1 promoters for certain breast cancers, and the α fetoprotein promoter of hepatomas. Enhancers may be used from viruses such as Rous sarcoma virus, hepatitis B virus, or simian virus-40 can be used. Enhancers from the cyclic AMP response element, serum response element, nuclear factor kappa b element, activator protein 1, or serum response factor may be used as well. Any enhancers known in the art can be used.

[35] The nucleic acids encoding human p53 may further comprise a selectable marker gene. The selectable marker gene allows easy detection of the transfer of the *p53* nucleic acids into a suitable host cell. Selectable marker genes may be genes that confer resistance to a toxic agent such as an antibiotic. Antibiotic resistance genes include those for ampicillin, tetracycline, puromycin, neomycin, and hygromycin. The selectable marker gene may confer resistance to a toxic agent; such genes include the adenine deaminase, aminoglycoside phosphotransferase, dehydrofolate reductase or xanthine-guanine phosphoribosyltransferase gene. The selectable marker gene may alternatively be a reporter gene whose expression is monitored readily by assay. Several nonlimiting examples of such reporter genes are chloramphenicol transacetylase, firefly luciferase, beta galactosidase, secreted alkaline phosphatase, and beta glucuronidase. The marker gene can also be a gene that allows growth of a cell on medium lacking an amino acid. An example of a selectable marker gene of yeast is the URA3 gene. Counterselectable genes can also be used with yeast such as LYS2, LYS5, CAN1, MET2, MET15, and GAL1. Other such marker genes are known in the art.

[36] The nucleic acids of the invention can also be isolated or in a cell. The cells can be of any type including mammalian cells, insect cells, *Drosophila* cells, yeast cells or bacterial cells. If the cells are mammalian cells, they can be the cells of any species including humans,

mice, monkeys, pigs, rats, cows, horses, cats, or dogs. The mammalian cells can further be manipulated to knock out one or both endogenous copies of the cell's *p53* genes encoded in its cellular DNA, thus allowing study of the alternative human *p53* nucleic acids alone in the cells.

[37] The mammalian cells can be in the body of a mammal or may be in *in vitro* culture. If the cells are in culture, the cells may be primary cells or may be a stable cell line. The cells may also contain a different wild type *p53* gene or may have a *p53* gene encoding a *p53* mutation that has been identified as being associated with a human cancer, a *p53* mutation that has not yet been associated with a human cancer, or a *p53* mutation that is not associated with a human cancer. The cells may also be tumor cells that may or may not contain a mutant *p53* gene. If the cells are in the body of a mammal the non-naturally occurring *p53* nucleic acids can be introduced as gene therapy to supply p53 activity or additional p53 activity to the cells.

[38] The human *p53* nucleic acids may be introduced into cells by any means known in the art. The nucleic acids may be inserted into the cells by direct transfer, by microinjecting the nucleic acids into the cells. The nucleic acids may also be complexed in a lipid preparation such as liposomes, or coacervated with a polymeric cation. The nucleic acids may alternatively be transferred into cells by electroporation, using DEAE dextran or calcium phosphate. The human *p53* nucleic acids may further be transferred into cells using viruses with suitable characteristics for entry into the cells. The transfer of the *p53* nucleic acids into the cells may achieve stable or transient transfection.

[39] The non-naturally occurring *p53* nucleic acids may be introduced into cells for expression and purification of p53 proteins. The purified human p53 protein may be used for crystallographic studies or for in vitro assays. Alternatively, the human p53 protein expressed from the non-naturally occurring nucleic acids may be assayed for activity in the cells. Yeast functional assays can be performed with the p53 expressed from the non-naturally occurring nucleic acids introduced into yeast (Brachmann et al., 1996; Vidal et al., 1996). Similarly mammalian cell assays have been developed for the study of p53 function. (Lowe et al., 1993b.)

[40] The non-naturally occurring nucleic acid molecule may additionally contain a *p53* mutation found in a human cancer. The mutation may be any *p53* mutation found in a human

cancer. Human cancers containing *p53* mutations include tumors of the digestive organs, respiratory system, breast, female genital organs, head and neck, hematopoeitic system, skin, brain, bladder, male genital organs, soft tissues, bone and others. Mutations of human p53 found in cancer include, but are not limited to: Lys132Arg; Cys135Tyr; Cys141Tyr; Pro151Ser; Gly154Val; Val157Phe; Arg158His; Arg158Leu; Ala161Thr; Tyr163Cys; Val173Leu; Val173Met; Arg175His; Cys176Phe; Cys176Tyr; His179Arg; His179Tyr; Ile195Thr; Tyr205Cys; His214Arg; Tyr220Cys; Tyr234Cys; Met237Ile; Cys238Tyr; Ser241Phe; Cys242Phe; Gly245Asp; Gly245Cys; Gly245Ser; Gly245Val; Arg248Gln; Arg248Leu; Arg248Trp; Arg249Met; Arg249Ser; Gly266Arg; Gly266Glu; Val272Met; Arg273Cys; Arg273His; Arg273Leu; Cys275Tyr; Pro278Leu; Pro278Ser; Arg280Lys; Arg280Thr; Asp281Glu; Arg282Trp; Glu285Lys; and Glu286Lys. Preferably the non-naturally occurring nucleic acid molecule containing a mutation found in a human cancer has a nucleotide sequence shown in one of SEQ ID NO: 4-53. It is also contemplated that the nucleic acid additionally contains a suppressor mutation of the *p53* mutation that is found in human cancer.

[41] Alternatively a non-naturally occurring nucleic acid molecule may contain a mutation in a codon for a residue that is post-translationally modified in wild-type p53. The mutation prevents posttranslational modification of the residue. The posttranslational modification may be phosphorylation, acetylation, sumoylation, or ubiquitylation.

[42] The residue modified by posttranslational modification may be any residue. If the posttranslational modification is phosphorylation, the residue modified may be any serine, threonine, or tyrosine. Preferably the residue modified is a serine at residue 6, 9, 15, 20, 33, 37, 46, 315, 371, 376, 378, or 392, or the threonine at residue 18 or 81. If the residue is modified by acetylation/deacetylation the residue is preferably a lysine at residue 320, 370, 372, 373, 381, or 382. If the residue is modified by sumoylation/desumoylation the residue is preferably the lysine at residue 386.

[43] While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the spirit and scope of the invention as set forth in the appended claims.

EXAMPLES

[44] Example 1. A new p53 open reading frame for identification and evaluation of intragenic suppressor mutations of the most common *p53* cancer mutations. Our previous study clearly established that various mechanisms for stabilizing the p53 core domain exist; and it is very likely that additional ones are waiting to be discovered (Brachmann et al., 1998). In light of the discovered *p53* suppressor mutations and the need for a comprehensive analysis for the most common *p53* cancer mutations, we have designed a much-improved strategy that will require more initial effort, but dramatically streamline future analyses. The design is based on several shortcomings that we encountered and includes the synthesis of a new *p53* ORF with multiple restriction sites, as well as specific codon choices for *p53* cancer mutations to allow for easy secondary screens.

[45] Once we had isolated the suppressor mutations in our prior study, we were very interested in establishing whether these mutations would be able to suppress other cancer mutations. This was fairly easily achieved for mutations such as T123A and T123P yet very cumbersome for N239Y, S240N and N268D due to the scarcity of naturally present restriction sites. We therefore synthesized an entirely new *p53* ORF that includes a multitude of unique restriction sites or sites with one additional site in the vector. The introduction of restriction sites by silent mutagenesis was performed using the program WebCutter 2.0 (http file type, www host server, firstmarket.com domain name, cutter/cut2.html directory) and the result is shown in Figure 2A.

[46] The new ORF was assembled from four fragments that were 379, 249, 257 and 325 base pairs long. Between 3 and 8 clones were sequenced for each of the *p53* ORF fragments. This identified clones without mutations for 2 of the 4 fragments. The third fragment could be constructed by combining the areas of two clones without mutations. The fourth fragment was constructed by repairing a single mutation with oligonucleotides. After assembling the entire new *p53* ORF with the ADH1-promoter and CYC1-terminator in pRS413 (CEN/HIS3), we compared the new pTW500 with the previously used p53 expression plasmid pRB16 (Figure 2).

[47] Both p53 expression plasmids showed the same phenotype in the presence of the p53-responsive URA3 reporter gene 1cUAS53::URA3 (Figure 2B). Four independent transformants were $Ura^{+}Foa^{S}$, while controls lacking either a p53 expression plasmid or the

reporter gene had the opposite phenotype, $Ura^{-}Foa^{R}$. All strains grew on SC-His plates, indicating the presence of the plasmid with the marker gene HIS3.

[48] Comparison of the growth rates in SC-Ura media confirmed the results of the plating assays: both plasmids lead to the expression of similar amounts of p53 protein (Figure 2C). Western Blot analysis using a monoclonal anti-p53 antibody showed that pTW500 indeed leads to p53 protein levels that are similar to and maybe slightly higher than those with pRB16 (Figure 2D).

[49] This new *p53* ORF is also optimized as much as possible for the preferential codon usage of *E. coli*, yeast and mammalian cells (Zhang et al., 1991; Wada et al., 1992). To make diagnostic restriction digestions easier to interpret, we also destroyed a variety of restriction sites.

[50] Example 2. Construction of expression cassettes for the most common mutated p53 proteins in humans. A comprehensive analysis is needed to better understand the structural dynamics of the p53 core domain and to potentially find suppressor mutations that have a universal effect. We chose to study the 50 most frequent *p53* cancer mutations. Based on several international databases for *p53* cancer mutations (Beroud and Soussi, 1998; Cariello et al., 1998; Hainaut et al., 1998), we initially selected a total of eight mutations (Figure 3). Each of these cancer mutations represents between 1.6% and 4.2% of all human cancers with *p53* mutations, totaling 22% (estimated to represent 73,000 cancer patients per year in the United States and 530,000 cancer patients worldwide), (Harris, 1996a, 1996b). Besides their high frequency, these eight cancer mutations also well represent the most important structural motifs of the p53 core domain (L2 loop: codon 175, L3 loop: codons 245, 248 and 249, loop-sheet-helix motif: codons 273 and 282).

[51] We further chose 27 mutations to examine in this final evaluation (Figure 3). Each cancer mutation in the second set accounts for 0.04 to 1.1% of reported *p53* mutations, totaling 15% of all human cancers with *p53* mutations (estimated to represent 50,000 cancer patients per year in the United States and 360,000 cancer patients worldwide), (Harris, 1996a, 1996b). This second set reflects many different mechanisms of destabilizing the p53 core domain since the mutations locate to different structural motifs (β sandwich: 8; loop-sheet-helix motif: 7; L2 loop: 5; L3 loop: 7).

[52] Example 3. Identification of suppressor mutations for the most common *p53* cancer mutations. New suppressor mutations have been isolated in the new *p53* ORF of plasmid pTW500. In order to isolate the new suppressor mutations alone, they will be subcloned into pTW500 and into the pCMVneo-based mammalian expression plasmid for *p53* (See Example 1). All subcloning steps will be confirmed either by sequencing or by verifying the loss or gain of a unique restriction site.

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[53] Transcriptional activity of wild-type p53 in yeast. This assay scores for the transcriptional activity of wild-type p53 and uses an artificial reporter gene, *1cUAS53::URA3*, with a synthetic consensus p53 DNA-binding site upstream of *URA3* (Brachmann et al., 1996; Vidal et al., 1996). Human *p53* is expressed from a yeast *CEN* expression plasmid under the control of the constitutive promoter *ADH1*. This p53 yeast assay is unique in that it not only allows selection for, but also against functional p53 (Brachmann and Boeke, 1997). Therefore, it can quickly classify any p53 protein for its activity: p53 proteins with wild-type p53 activity induce *URA3* expression which enables the yeast reporter strain to survive on plates without Uracil (Ura^+), but also sensitizes the strain to 5-fluoro-orotic acid (5-Foa) resulting in the second phenotype, Foa sensitivity (Foa^S). P53 cancer mutants that have lost function have the opposite phenotype of wild-type p53, Ura^- Foa^R (Brachmann et al., 1996). p53 proteins with partial loss of wild-type p53 function can be easily detected as well since they have the intermediate Ura^+Foa^R phenotype. This reflects sufficient *URA3* expression for survival on SC -Ura plates, but insufficient expression to be sensitive to 5-Foa.

[54] Engineered *p53* open reading frame. A new ORF for wild-type p53 with multiple silent restriction sites and optimized for codon usage in bacteria, yeast and mammalian cells (Wada, 199; Zhang, 1991) was designed with WebCutter 2.0 (http file type, www host server, firstmarket.com domain name, cutter/cut2.html directory) and constructed using the "KAPPA" method (Holowachuk, 1995). When compared with the natural *p53* yeast expression plasmid pRB16, the engineered *p53* ORF in the same yeast plasmid resulted in two-fold more p53 protein and identical yeast phenotypes (Brachmann, 1996; Brachmann, 1998; Vidal, 1996). Annealed oligonucleotides encoding for the most common *p53* cancer mutations (see Table 2) were cloned into the engineered *p53* ORF.

[55] PCR- and oligonucleotide-mediated mutagenesis strategy to identify intragenic suppressor mutations for *p53* cancer mutations. PCR-mediated mutagenesis was performed as previously described (Brachmann, 1998), except that engineered *p53* ORFs for the *p53* cancer mutants, mutagenic PCR conditions (Lin-Goerke, 1997; Svetlov, 1998) and Rby377, a diploid yeast strain with two copies of the p53-dependent *URA3* reporter gene, were used. A library of annealed oligonucleotides that equally represented all possible amino acid changes in codons 239 and 240 and had approximately one in 100 additional nucleotides

mutated were cloned into PflMI-NsiI gapped yeast expression plasmids for *p53* cancer mutants, transformed into Rby377 and analyzed as described (Brachmann, 1998).

[56] Yeast and mammalian assays for p53. Yeast assays for p53 and mammalian reporter gene assays were performed as described (Brachmann, 1996; Brachmann, 1998; Vidal, 1996).

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